CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

BIOLOGIC BYPASS WITH THE USE OF ADENOVIRUS-MEDIATED GENE TRANSFER OF THE COMPLEMENTARY DEOXYRIBONUCLEIC ACID FOR VASCULAR ENDOTHELIAL GROWTH FACTOR 121 IMPROVES MYOCARDIAL PERFUSION AND FUNCTION IN THE ISCHEMIC PORCINE HEART

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Objectives: **Vascular endothelial growth factor (VEGF), a potent angiogenic mediator, can be delivered to targeted tissues by means of a replication-deficient adenovirus (Ad) vector. We hypothesized that direct administration of Ad vector expressing the $VEGF_{121}$ complementary deoxyribonucleic acid (Ad_{GV}VEGF121.10) into regions of ischemic myocardium would enhance collateral vessel formation and improve regional perfusion and function.** ***Methods:*** **Yorkshire swine underwent thoracotomy and placement of an Ameroid constrictor (Research Instruments & MFG, Corvallis, Ore.) on the circumflex coronary artery. Three weeks later, myocardial perfusion and function were assessed by single photon emission computed tomography imaging (SPECT) with ^{99m}Tc-labeled sestamibi and by echocardiography during rest and stress. Ad_{GV}VEGF121.10 ($n = 7$) or the control vector, AdNull ($n = 8$), was administered directly into the myocardium at 10 sites in the circumflex distribution (10^8 pfu/site). Four weeks later, these studies were repeated and ex vivo angiography was performed.** ***Results:*** **SPECT imaging 4 weeks after vector administration demonstrated significant reduction in the ischemic area at stress in Ad_{GV}VEFG121.10-treated animals compared with AdNull control animals ($p = 0.005$). Stress echocardiography at the same time demonstrated improved segmental wall thickening in Ad_{GV}VEGF121.10 animals compared with AdNull control animals ($p = 0.03$), with Ad_{GV}VEGF121.10 animals showing nearly normalized function in the circumflex distribution. Collateral vessel development assessed by angiography was also significantly greater in Ad_{GV}-VEGF121.10 animals than in AdNull control animals ($p = 0.04$), with almost complete reconstitution of circumflex filling in Ad_{GV}VEGF121.10 animals.** ***Conclusions:*** **An Ad vector expressing the $VEGF_{121}$ cDNA induces collateral vessel development in ischemic myocardium and results in significant improvement in both myocardial perfusion and function. Such a strategy may be useful in patients with ischemic heart disease in whom complete revascularization is not possible. (J Thorac Cardiovasc Surg 1998;115:168-77)**

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Despite continued advances in the treatment of ischemic heart disease, a large population of individuals with diffuse coronary artery disease exists for whom conventional therapies such as percutaneous angioplasty and bypass surgery provide little or no benefit. A new strategy that may be applicable to such patients is "therapeutic angiogenesis," the induction of new blood vessel development into areas with limited blood flow.[1, 2] Therapeutic angiogenesis capitalizes on the discovery of protein mediators that elicit angiogenesis, a complex process that includes the migration and proliferation of endothelial cells, vascular tube formation, and linkage to the preexisting vascular network.[3, 4] Several of these protein mediators have been shown to promote revascularization of ischemic tissues in models of myocardial or peripheral ischemia.[1-3, 5-9]

Vascular endothelial growth factor (VEGF), a homodimeric 34 to 46 kDa heparin-binding glycoprotein, is the most specific of the known angiogenic mediators because of localization of its receptors almost exclusively on endothelial cells.[10-12] The human VEGF gene is expressed as four isoforms secondary to posttranscriptional splicing, producing proteins of 121, 165, 189, and 206 residues. The observations that VEGF and VEGF receptors (flk-1/KDR and flt-1) are up-regulated under ischemic conditions is consistent with the concept that VEGF is an endogenous mediator of angiogenesis.[11-13]

Gene therapy, in which the complementary deoxyribonucleic acid (cDNA) for VEGF is delivered directly to tissues, has the potential to induce localized VEGF expression that is of limited duration, thus avoiding the toxicity and promiscuous angiogenesis potentially associated with systemic protein therapy.[14-18] In examining this hypothesis, the present study uses a porcine myocardial ischemia model to demonstrate that direct administration of a replication-deficient adenovirus (Ad) vector coding for the human $VEGF_{121}$ cDNA (Ad_{GV}-VEGF121.10) into ischemic myocardium induces a "biologic bypass"—collateralization around a site of coronary occlusion, with concomitant improvement in regional myocardial perfusion and function during stress-induced myocardial ischemia.

Methods

Experimental model of myocardial ischemia. A model of chronic myocardial ischemia was created in Yorkshire swine (28 to 30 kg). All animal care procedures were in accordance with institutional guidelines. Animals were sedated with intramuscular tiletamine and zolazepam (Telazol, 3.3 mg/kg) and xylazine (0.10 mg/kg), intubated, and sedation was maintained with 0.5% to 2.0% isoflurane. A limited left thoracotomy was performed in a sterile fashion through the fifth intercostal space and a small incision was made in the pericardium. A 2.5 mm internal diameter Ameroid constrictor (Research Instruments & MFG, Corvallis, Ore.) was placed around the circumflex artery as proximally as possible. Topical lidocaine 1% solution was applied to the circumflex artery at the Ameroid constrictor site to prevent coronary artery spasm. The pericardium and chest were then closed and the animal was allowed to recover.

Ad vectors. The replication-deficient vector Ad_{GV}-VEGF121.10 is an $E1a^-$, partial $E1b^-$, partial $E3^-$Ad vector that contains an expression cassette in the E1 position (right to left) containing the cytomegalovirus immediate early promoter/enhancer, an artificial splice sequence, the human $VEGF_{121}$ cDNA, and the SV40 polyA/stop signal. AdNull (similar to Ad_{GV}VEGF121.10, but with no gene in the expression cassette) was used as a control vector.[19] All Ad vectors were propagated and titrated in 293 cells, purified by cesium chloride density purification, dialyzed, and stored at –70° C.[20] The viral stocks were demonstrated to be free of replication-competent wild type Ad. Biologic activity of the $VEGF_{121}$ transgene product was confirmed by demonstrating proliferation of human umbilical vein endothelial cells using [^{3}H]thymidine incorporation, and in vivo confirmation of transgene expression was determined by enzyme-linked immunosorbent assay analysis of myocardial biopsy specimens obtained from AdVEGF121.10 injection sites 3 days after vector administration.[17]

In vivo administration of Ad vectors. Three weeks after Ameroid constrictor placement, the left thoracotomy was reopened and administration of the therapeutic vector, Ad_{GV}VEGF121.10, or the control vector, AdNull, was performed by direct myocardial injection (Fig. 1). Each vector was injected at 10 sites, each in 100 μl phosphate-buffered saline solution, pH 7.4, in the circumflex distribution (10^8 pfu/injection). Pacing wires were placed in the left atrial appendage and tunneled subcutaneously for subsequent stress technetium 99m (^{99m}Tc)-labeled sestamibi (Cardiolite, DuPont Pharma, N. Billerica, Mass.) assessment of regional myocardial perfusion by single photon emission computed tomography (SPECT) and echocardiographic assessment of regional wall thickening.

^{99m}Tc-labeled sestamibi assessment of regional myocardial perfusion. Regional myocardial perfusion was evaluated during rest and stress 3 weeks and 7 weeks after placement of the Ameroid constrictor by means ^{99m}Tc-sestamibi SPECT. During rapid atrial pacing at 200 beats/min, animals received intravenous injections of a 5 mCi bolus of ^{99m}Tc-sestamibi and pacing was continued for approximately 3 minutes. The animals were then placed in the prone position in an ADAC Vertex dual detector gamma camera system (ADAC Laboratories, Milpitas, Calif.). A nongated SPECT study was then acquired in a "step-and-shoot" mode over a 180-degree body-contouring orbit. The animal was allowed to return to baseline heart rate and was then received an injection of a 25 mCi bolus of ^{99m}Tc-sestamibi before obtaining a rest SPECT, acquired in an analogous fashion.

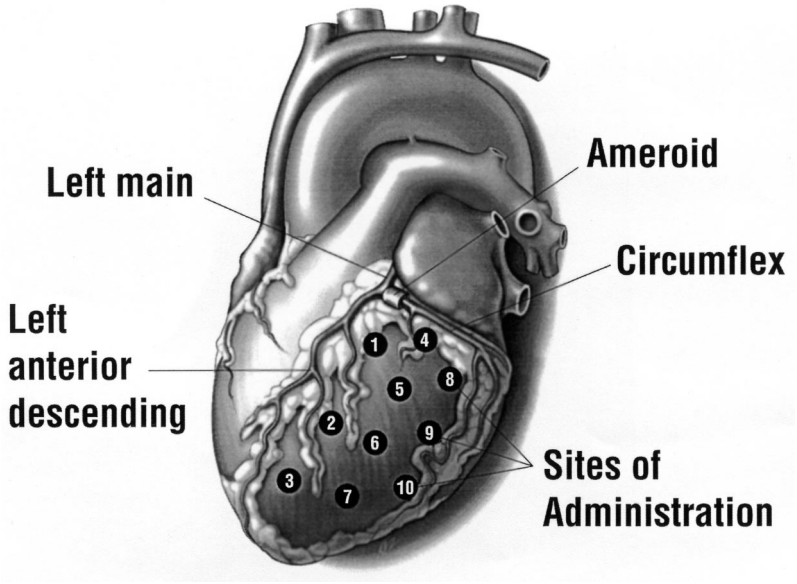

Fig. 1. Schema of the experimental design. Three weeks after Ameroid constrictor placement, animals were administered either $Ad_{GV}VEGF121.10$ ($n = 7$) or the control vector AdNull ($n = 8$) at 10 sites (10^8 pfu/site) distributed throughout the left ventricular region between the circumflex and left anterior descending coronary arteries, as indicated.

The rest and stress SPECT studies were processed in a blinded fashion with the use of an integrated ADAC Pegasys computer. Stress and rest circumferential count profiles (polar plots) at the midventricular level were constructed by dividing the midventricular short-axis image into 60 angular segments centered on the ventricular cavity, determining the number of counts per segment, normalizing the number of counts in each segment to the segment with the maximum number of counts (assigned a reference value of 100), and plotting the normalized counts per segment versus the angular position of the segment. The polar plots were transferred to ASCII files for further analysis with the program SIGMAPLOT (Jandel Scientific, Corte Madera, Calif.).

For each animal, the extent of myocardial ischemia ("area") was determined from the difference between the rest and stress polar plots. The maximum severity of ischemia ("ischemia maximum") in the circumflex distribution was determined by ascertaining the point of greatest difference between the rest and stress plots and measuring the difference in the plots at that point. The percent improvement in myocardial perfusion for each animal was calculated for these two parameters as ("parameter" at 3 weeks − "parameter" at 7 weeks × 100)/("parameter" at 3 weeks).

Echocardiographic assessment of regional myocardial function. Baseline regional myocardial function was assessed by echocardiography at rest and during stress at the time of vector administration. Animals were sedated and placed in the left lateral decubitus position, and standard two-dimensional and M-mode transthoracic images were obtained with an HP2500 echocardiographic machine and a 3.0/3.5 MHz dual-frequency transthoracic transducer (Hewlett-Packard, Andover, Mass.). From the right parasternal approach, short-axis, midpapillary views were obtained at rest for 3 minutes. The animals then underwent rapid left atrial pacing in a stepwise fashion to the target ventricular rate of 200 beats/min, at which time imaging was recorded for an additional 3 minutes.

Regional wall thickening was determined by a single experienced investigator in a blinded fashion, tracing the endocardial and epicardial surfaces of the left ventricle in both diastole and systole using a Digisonics CardioRevue System (Digisonics Inc., Houston, Tex.). Systolic wall thickening in each of six equal radial 60-degree segments was defined as mean systolic wall thickness − mean diastolic wall thickness. Fractional wall thickening was calculated as mean systolic wall thickening/mean diastolic wall thickness. The ischemic and nonischemic zones for each animal were defined from rapid atrial pacing images at 3 weeks (baseline ischemia) as the two contiguous segments with the lowest and highest fractional wall thickening, respectively. This corresponded in all cases with the circumflex region and the septum, respectively. The same zones for each animal were analyzed in rapid atrial pacing images at 7 weeks.

Ex vivo coronary angiography. When each animal was put to death (4 weeks after vector administration), the heart was arrested with 40 mEq of KCl and then perfusion-fixed at 100 mm Hg with 1 L of McDowell-Trump fixative (4% formaldehyde, 1% glutaraldehyde, 1% NaH_2PO_4 and 0.3% NaOH adjusted to pH 7.2). Ex vivo coronary angiography was performed by the same angiographer in a blinded fashion using a 5F end-hole wedge balloon catheter (Arrow Inc., Reading, Pa.) placed in the left main coronary artery. By means of cinefluoroscopy in

Table I. *Quantitative assessment of regional stress-induced myocardial ischemia by ^{99m}Tc-labeled sestamibi SPECT imaging*

	*Ischemic area**			*Ischemic maximum**		
Time of study	*Ad_{GV}VEGF121 (n = 8)*	*AdNull (n = 7)*	*p Value*	*Ad_{GV}VEGF121 (n = 8)*	*AdNull (n = 7)*	*p Value*
3 wk (vector administered)	3570 ± 640	3200 ± 390	0.8	30.7 ± 3.6	26.8 ± 2.1	0.2
7 wk	750 ± 220	2140 ± 400	0.005	11.0 ± 2.2	22.3 ± 1.7	0.04

*Values are mean ± standard error of the mean; regional stress-induced was quantified by determining the area of ischemia and ischemia maximum as described in Fig. 2, *A* and the *Methods* section.

the standard right anterior oblique projection with continuous image acquisition, 5 ml of contrast medium (Hypaque-76, Nycomed Inc., New York, N.Y.) was injected at a continuous rate until the entire left anterior descending coronary artery and its branches were completely opacified.[21] Collateral vessels from the left anterior descending coronary artery, which reconstituted the circumflex coronary artery or obtuse marginal branch of the circumflex coronary artery, were quantified by three blinded observers using the grading method of Rentrop and associates[22] as follows: 0 = no filling of collateral vessels; 1 = filling of collateral branches of the circumflex or obtuse marginal branch without visualization of the epicardial segment; 2 and 3 = partial or complete filling of the epicardial segment of the circumflex or obtuse marginal artery via collateral vessels, respectively.

Histologic evaluation. After angiography, the left ventricle of each heart was sectioned into three rings in the short axis. Forty 5 μm histologic sections from each heart were taken at equidistant intervals around the basal and midventricular rings, processed through paraffin, and stained with hematoxylin and eosin. Histologic evidence of infarction and inflammation for each tissue section was graded by a pathologist blinded to treatment on a scale of 0 to 4 as follows: 0 = none; 1 = one to three small areas involved; 2 = less than 10% section surface; 3 = more than 10% up to 50% section surface; and 4 = more than 50% section surface.

Statistical analysis. Treatment was assigned in an alternating consecutive fashion to a total enrollment of 15 animals that could be evaluated for efficacy, with myocardial ischemia area defined as the primary outcome variable. Statistical analysis was carried out by means of the Mann-Whitney nonparametric test. All results are expressed as mean ± standard error of the mean.

Results

Overall assessment. All of the 19 animals entered into the study (Ad_{GV}VEGF121.10, n = 9; AdNull, n = 10) survived until put to death 7 weeks after placement of the Ameroid constrictor, without clinical evidence of toxicity. At 3 weeks (i.e., before therapy), four of the 19 pigs (Ad_{GV}VEGF121.10, n = 2; AdNull treated, n = 2) had evidence of myocardial infarction in the circumflex region, as demonstrated by (1) a fixed defect (no difference between rest and stress) in the circumflex zone of the ^{99m}Tc-sestamibi SPECT images and (2) a thinned, akinetic posterolateral region of the left ventricle in short-axis views during echocardiography at rest. Consistent with the ^{99m}Tc-sestamibi SPECT and echocardiography suggesting myocardial infarction 3 weeks after Ameroid constrictor placement, the gross pathologic evaluation 4 weeks later showed myocardial scarring and thinning of at least 25% of the total ventricular mass. All four pigs in this subgroup also had histologic evidence of large transmural infarction. On the basis of these data, these four animals were excluded from further analysis. Thus the group of animals evaluated for efficacy of therapy included seven Ad_{GV}-VEGF121.10-treated animals and eight AdNull (control) animals.

Table II. *Stress-induced regional contractile dysfunction assessed by two-dimensional echocardiography*

	*Difference in fractional wall thickening (nonischemic zone − ischemic zone, %)**		
Time of study	*Ad_{GV}VEGF121 (n = 8)*	*AdNull (n = 7)*	*p Value*
3 wk (vector administered)	16 ± 4.2	17.2 ± 4.4	0.9
7 wk	−0.06 ± 3.3	12.4 ± 4.0	0.03

Values are mean ± standard error of the mean; Short-axis images of two-dimensional echocardiograms were divided into six radial segments, and the fractional wall thickening (systolic wall thickening as a percentage of diastolic wall thickness) of the ischemic zones was assessed as described in the *Methods* section. The percent difference in fractional wall thickening of the nonischemic zone minus the ischemic zone was calculated for AdNull and Ad_{GV}VEGF121.10; zero difference in fractional wall thickening signifies equivalent function in the ischemic and nonischemic zones.

In vivo expression of the Ad_{GV}VEGF.10 vector was confirmed by demonstrating local myocardial VEGF expression after myocardial injection of 10^8 pfu of Ad_{GV}VEGF121.10 (n = 3). Three days after administration of the vector, myocardial levels were 0.75 ± 0.25 ng/mg protein, compared with back-

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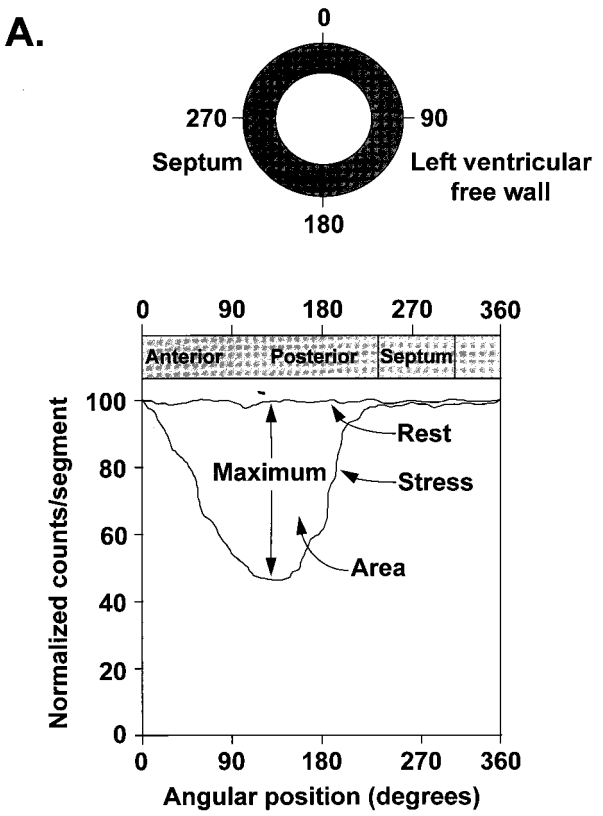

Fig. 2. Quantitative assessment of regional stress-induced myocardial ischemia with ^{99m}Tc-labeled sestamibi SPECT imaging. **A,** Schema of method using circumferential count profiles. Short-axis ^{99m}Tc-sestamibi images at the midventricular level were analyzed as described in the *Methods* section. The extent and severity (area) of myocardial ischemia was determined from the difference between the rest and stress circumferential count profile curves. The greatest severity of ischemia (ischemia maximum) in the circumflex distribution was determined as the greatest difference between the rest and stress circumferential count profiles.

ground expression in AdNull-treated animals ($p = 0.01$).

Ad_{GV}VEGF121.10-mediated increase in myocardial perfusion. Circumferential count profiles (polar plots) of ^{99m}Tc-sestamibi SPECT data from the midventricular level were used to quantify (1) the extent and severity of ischemia ("area") and (2) the most severe ischemia ("ischemia maximum") (Fig. 2, *A).* Circumferential plots of rest images obtained at 3 weeks typically demonstrated minimal perfusion defects, compared with plots of stress (pacing) images, which revealed decreased perfusion in the posterolateral region, corresponding to the occluded circumflex coronary artery distribution (Fig. 2, *B* and *D).* The ischemic area and ischemia maximum were characteristically unchanged from baseline in AdNull animals assessed 4 weeks after vector administration (Fig. 2, *B* and *C).* In contrast, Ad_{GV}VEGF121.10 animals demonstrated improvement in myocardial perfusion 4 weeks after vector administration, as demonstrated by decreases in the ischemic area and ischemic maximum compared with baseline (Fig. 2, *D* and *E).* Corresponding changes were noted at the apical, midventricular, and basal levels.

The ischemic area was similar in both the Ad_{GV}VEGF121.10 and AdNull control animals at the time of vector administration (Table I). In contrast, the ischemic area was significantly reduced at 7 weeks in the Ad_{GV}VEGF121.10 animals compared with the AdNull animals. The "percent improvement" in the area of ischemia of each animal 4 weeks after vector administration, compared with baseline, was approximately 2.4-fold greater in the Ad_{GV}VEGF121.10 animals than in the AdNull animals (75% ± 6% vs 32% ± 11%, respectively, $p = 0.01$).

The ischemia maximum in the circumflex distribution was also the same for the Ad_{GV}VEGF121.10 animals and AdNull control animals at 3 weeks (Table I). In contrast, 4 weeks after vector administration, the ischemia maximum was significantly decreased in the Ad_{GV}VEGF121.10 animals than in the AdNull control animals. Similarly, the "percent improvement" in the ischemia maximum was 2.5-fold greater in Ad_{GV}VEGF121.10 animals than in the AdNull control animals (56% ± 8% vs 22% ± 6%, $p = 0.01$).

Ad_{GV}VEGF121.10-mediated improvement in myocardial function. Three weeks after Ameroid constrictor placement, myocardial function in the ischemic circumflex region compared with the nonischemic septum was similar in the Ad_{GV}VEGF121.10 group compared with AdNull controls as assessed by fractional wall thickening during rapid atrial pacing (Table II). In contrast, by 4 weeks after vector administration, Ad_{GV}VEGF121.10-treated animals demonstrated significantly greater improvement in fractional wall thickening during rapid atrial pacing than did AdNull control animals. Strikingly, contractile function in the circumflex segment of the Ad_{GV}VEGF121.10 group approximated that of the septal (control) segment, as reflected by an ischemic minus nonischemic zone difference of "zero" in this analysis.

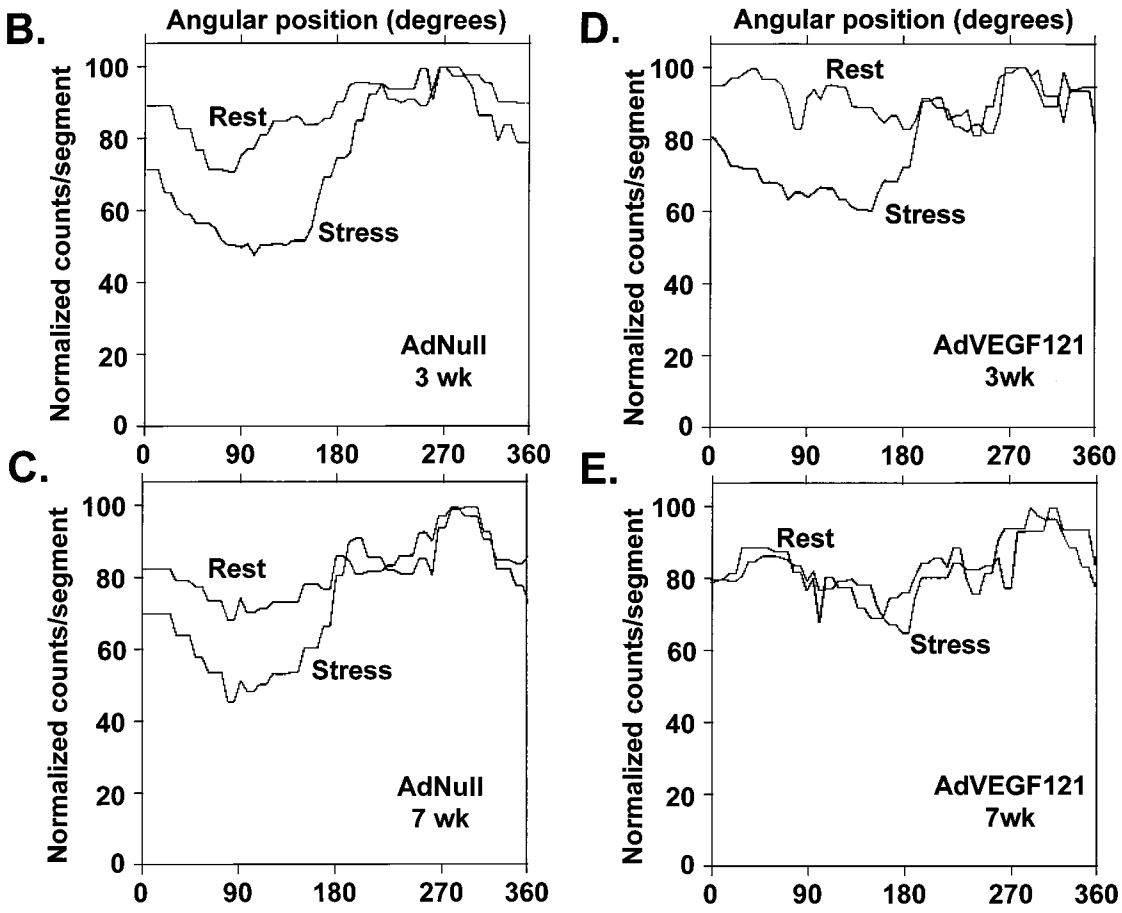

Fig. 2. Cont'd. **B,** Representative circumferential count profiles of an AdNull-treated animal at 3 weeks (at the time of vector administration) at rest *(upper part of the panel)* and stress (atrial pacing; *at the lower part of the panel),* showing a perfusion defect in the posterolateral region. **C,** Same animal as in panel **B,** but at 7 weeks. There is minimal decrease in area of ischemia and ischemia maximum compared with 3 weeks. **D,** Representative circumferential count profiles of an Ad_{GV}VEGF121-treated animal at 3 weeks (at the time of vector administration) at rest and stress (atrial pacing), showing a perfusion defect in the posterolateral region. **E,** Same animal as in panel **D,** but at 7 weeks; there is a marked decrease in both area of ischemia and ischemia maximum compared with observations at 3 weeks.

Angiographic assessment of coronary vessels. Ex vivo angiography performed 4 weeks after vector administration confirmed complete occlusion of the proximal circumflex coronary artery by the Ameroid constrictor in all animals. AdNull-treated animals characteristically demonstrated only partial filling of the obtuse marginal and circumflex coronary arteries (Fig. 3, *A).* In contrast, animals that received Ad_{GV}VEGF121.10 typically demonstrated nearly complete reconstitution of both the obtuse marginal and circumflex coronary circulations (Fig. 3, *B* and *C).*

The collateral grade for the obtuse marginal and circumflex coronary arteries was significantly greater in the Ad_{GV}VEGF121.10 animals than in the AdNull animals (Table III). Finally, the total number of angiographically visible collateral vessels filling the circumflex and obtuse marginal arteries was significantly greater in the Ad_{GV}VEGF121.10 animals than in the AdNull animals (Table III).

Histologic assessment. The myocardium in 13 of the 15 animals in the study was available for assessment of inflammation (Ad_{GV}VEGF121.10, $n = 5$;

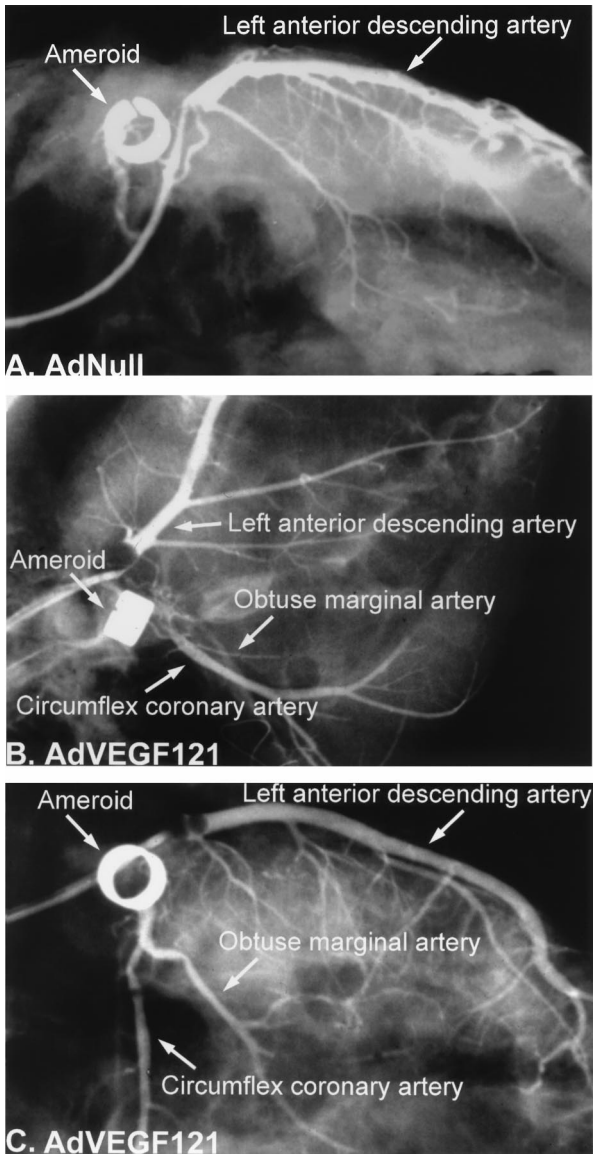

Fig. 3. Representative ex vivo angiograms of pig hearts 7 weeks after placement of Ameroid constrictors. The vectors were administered 3 weeks after placement of the Ameroid constrictor. **A,** AdNull. **B** and **C,** Ad_{GV}-VEGF121.10 (referred to as AdVEGF121). The Ameroid constrictor completely occludes the circumflex artery in both the AdNull and AdVEGF121-treated animals. The AdNull-treated animal demonstrates only minimal filling of the distal circumflex artery. In contrast, the AdVEGF121-treated animals show nearly complete reconstitution of the circumflex artery.

AdNull, $n = 8$). Minimal inflammation was detected in the myocardium of these animals evaluated 4 weeks after therapy, with no difference in the extent of inflammation between the Ad_{GV}VEGF121.10 and AdNull groups (overall intensity score 0.3 ± 0.06 *vs* 0.4 ± 0.08, $p = 0.4$).

Table III. *Quantitative assessment of collateral vessel grade and number as assessed by ex vivo angiography*

	Ad_{GV}*VEGF121** (*n* = 8)	*AdNull** (*n* = 7)	*p Value*
Collateral grade (scale: 0-3)			
To obtuse marginal	2.9 ± 0.1	1.8 ± 0.2	0.03
To circumflex	2.8 ± 0.1	1.9 ± 0.3	0.04
Collateral vessel number†	4.0 ± 0.4	2.0 ± 0.3	0.001

*Values are mean ± standard error of the mean determinations of three blinded observers as described in the *Methods* section.

†Number of angiography-visible collateral vessels observed between the left anterior descending and the circumflex or obtuse marginal arteries.

Discussion

This study demonstrates that Ad-mediated transfer of the cDNA of human VEGF isoform 121 directly into the myocardium of Yorkshire swine with an occluded circumflex coronary artery results in significant and physiologically relevant improvement in regional myocardial perfusion and contractile function during stress-induced myocardial ischemia. Importantly, this improvement was associated with increased myocardial collateral vessel development, "biologically bypassing" the experimentally occluded coronary artery segment.

VEGF as a mediator of therapeutic angiogenesis. Considerable information is available suggesting that the VEGF protein can induce angiogenesis in a variety of animal models of ischemia.[5-9, 15] In regard to cardiac ischemia, a single intracoronary bolus of $VEGF_{165}$ improves blood flow to the ischemic region,[9] and continuous infusion of the $VEGF_{165}$ protein via indwelling catheters increases collateral blood flow to ischemic myocardium and the numeric density of intramyocardial distribution vessels.[5] Finally, continuous administration of $VEGF_{165}$ to the surface of the myocardium over 6 weeks reduces the ischemic zone and improves ejection fraction and regional myocardial wall thickening as assessed by magnetic resonance imaging.[8]

Our choice of VEGF for this study is based on these findings and the endothelial cell specificity of VEGF, which offers potential advantages over the use of other growth factors with angiogenic properties (such as the fibroblast growth factors), which are also mitogenic for cells such as fibroblasts and smooth muscle cells, and thus theoretically impose the risk of unwanted fibrosis or

smooth muscle cell hyperplasia.[3, 23, 24] Although the major VEGF isoforms appear to be equipotent in angiogenic potential, our choice of the cDNA coding for the 121 amino acid isoform of VEGF was based on the knowledge that the $VEGF_{121}$ isoform does not bind heparin and thus may diffuse throughout the myocardium more readily than the other isoforms.[11, 12]

Ad-mediated gene transfer as a strategy for VEGF-related therapeutic angiogenesis. Gene therapy holds several potential advantages over a protein-based strategy for therapeutic angiogenesis. Gene transfer provides the equivalent of a "slow-release depot," providing high concentrations of the therapeutic protein for a relatively extended period. Ad vector expressing VEGF provides myocardial expression of VEGF protein for up to 7 days.[17] In contrast, the VEGF protein has a very short biologic half-life in the circulation,[6] and most previous studies have consequently required continuous infusions or repetitive dosing of the growth factor to achieve a therapeutic effect.[5, 8, 15] Another potential advantage of gene therapy over protein-mediated angiogenic therapy is that gene transfer can be strategized to provide delivery of a high concentration of VEGF localized to the ischemic sites. In contrast, systemic administration of a single large bolus of VEGF protein can cause hypotension, and systemic therapy carries the theoretic risk of inducing inappropriate angiogenesis at sites of vascular derangement, or at sites where angiogenesis might have major adverse consequences, such as the retina, the synovium, and in occult tumors.[3, 9, 11, 12, 16]

Although three gene transfer systems—naked plasmids, herpes simplex virus, and Ad—have been used to induce angiogenesis with VEGF in experimental models, we have focused on Ad vectors because their inherent properties make them ideal for use in therapeutic angiogenesis. Ad vectors achieve gene transfer in both dividing and nondividing cells, with high levels of protein expression in cardiovascular relevant sites, such as myocardium, vascular endothelium, and skeletal muscle.[14, 17, 25, 26] The new gene transferred by an Ad vector functions in an epi-chromosomal position and thus carries little risk of inappropriately inserting the newly transferred gene in a critical site of the genome.[27] Most important, whereas long-term expression of angiogenic proteins might induce excessive, disorganized blood vessel formation, Ad vectors are highly efficient at achieving high levels of gene expression in cardiovascular tissues for only 1 to 2 weeks, thus limiting expression to that necessary to induce angiogenesis.[14, 17, 18] Consistent with the concept that Ad vectors can be used to deliver genes relevant to inducing myocardial angiogenesis, an Ad vector has been used to deliver the fibroblast growth factor–5 cDNA to the ischemic porcine myocardium, with resulting increases in perfusion, function, and histologic evidence of angiogenesis.[14]

Host immune response is a potential disadvantage to the use of Ad vectors in some gene transfer applications. With the use of clinical grade vectors, as in the present study, however, inflammation does not appear to be a significant problem. The cellular immune response that likely limits vector expression is actually an advantage for the application of therapeutic angiogenesis, as previously discussed. Finally, although it is unlikely that repeated administrations will be needed, the theoretic development of neutralizing antibodies directed against the Ad vector that might limit the effectiveness of repeated vector administrations can likely be overcome by the use of Ad vectors of different serotypes or by immunosuppressive therapy.[28]

Ad-mediated angiogenesis as a biologic revascularization strategy. The primary significance of the present results is that a one-time direct myocardial administration of an Ad vector containing the $VEGF_{121}$ cDNA induces the development of collateral vessels adequate to enhance myocardial perfusion and ameliorate regional myocardial contractile dysfunction in the setting of stress-induced myocardial ischemia. The observation in control animals of some collateral vessel development is consistent with evidence of the limited, incomplete nature of the endogenous processes of angiogenesis and collateralization that are known to occur in the setting of ischemia. In this context, the therapeutic angiogenesis described in this study can be considered as a logical enhancement of the normal, endogenous response to ischemia. With the evidence that Ad vectors are capable of delivering new genetic information safely to human beings in vivo,[27] the clinical applications of this strategy include its use as a novel therapeutic "biologic bypass" in patients with coronary disease.

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REFERENCES

1. Engler DA. Use of vascular endothelial growth factor for therapeutic angiogenesis. Circulation 1996;94:1496-8.
2. Simons M, Ware JA. Food for starving hearts. Nature Med 1996;2:519-20.
3. Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267: 10931-4.
4. Schaper W, Ito WD. Molecular mechanisms of coronary collateral vessel growth. Circ Res 1996;79:911-9.
5. Banai S, Jaklitsch MT, Shou M, et al. Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. Circulation 1994;89:2183-9.
6. Takeshita S, Zheng LP, Brogi E, et al. Therapeutic angiogenesis: a single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. J Clin Invest 1994;93:662-70.
7. Asahara T, Bauters C, Zheng LP, et al. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. Circulation 1995;92(Suppl): II365-71.
8. Pearlman JD, Hibberd MG, Chuang ML, et al. Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. Nature Med 1995;1:1085-9.
9. Hariawala MD, Horowitz JJ, Esakof D, et al. VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. J Surg Res 1996;63:77-82.
10. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989;246:1306-9.
11. Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocrinol Rev 1992;13:18-32.
12. Thomas KA. Vascular endothelial growth factor, a potent and selective angiogenic agent. J Biol Chem 1996;271:603-6.
13. Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E. Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis. Cardiovasc Res 1994;28:1176-9.
14. Giordano FJ, Ping P, McKirnan MD, et al. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. Natl Med 1996;2:534-9.
15. Harada K, Friedman M, Lopez JJ, et al. Vascular endothelial growth factor administration in chronic myocardial ischemia. Am J Physiol 1996;270:H1791-802.
16. Yang RH, Thomas GR, Bunting S, et al. Effects of vascular endothelial growth factor on hemodynamics and cardiac performance. J Cardiovasc Pharmacol 1996;27:838-44.
17. Magovern CJ, Mack CA, Zhang J, et al. Direct in vivo gene transfer to canine myocardium using a replication-deficient adenovirus vector. Ann Thorac Surg 1996;62:425-34.
18. Magovern CJ, Mack CA, Zhang J, Rosengart TK, Isom OW, Crystal RG. Regional angiogenesis induced in non-ischemic tissue by an adenovirus vector expressing vascular endothelial growth factor. Hum Gene Ther 1997;8:215-27.
19. Hersh J, Crystal RG, Bewig B. Modulation of gene expression after replication-deficient, recombinant adenovirus-mediated gene transfer by the product of a second adenovirus vector. Gene Ther 1995;2:124-31.
20. Rosenfeld MA, Yoshimura K, Trapnell BC, et al. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell 1992;68: 143-55.
21. Lee CC, Aruny JE, Laurence RG, Appleyard RF, Couper GS, Cohn LH. Bench coronary angiography: a potentially useful method to assess coronary artery disease in the older donor heart without catheterization laboratory angiography. J Heart Lung Transplant 1992;11:693-7.
22. Rentrop KP, Cohen M, Blanke H, Phillips RA. Changes in collateral channel filling immediately after controlled coronary artery occlusion by an angioplasty balloon in human subjects. J Am Coll Cardiol 1985;5:587-92.
23. Banai S, Jaklitsch MT, Casscells W, et al. Effects of acidic fibroblast growth factor on normal and ischemic myocardium. Circ Res 1991;69:76-85.
24. Pusztai L, Lewis CE, Lorenzen J, McGee JO. Growth factors: regulation of normal and neoplastic growth. J Pathol 1993; 169:191-201.
25. Lemarchand P, Jones M, Yamada I, Crystal RG. In vivo gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. Circ Res 1993;72:1132-8.
26. Kass-Eisler A, Falck-Pedersen E, Alvira M, et al. Quantitative determination of adenovirus-mediated gene delivery to rat cardiac myocytes in vitro and in vivo. Proc Natl Acad Sci U S A 1993;90:11498-502.
27. Crystal RG. Transfer of genes to humans: early lessons and obstacles to success. Science 1995;270:404-10.
28. Mack CA, Song W, Carpenter H, et al. Circumvention of anti-adenovirus neutralizing immunity by administration of an adenovirus vector of an alternate serotype. Hum Gene Ther 1997;8:99-109.

Discussion

Dr. Andrew S. Wechsler *(Richmond, Va.).* This work demonstrates the efficacy of Ad-based gene transfer as a means to achieve a potentially clinically applicable treatment strategy. It far transcends studies that have focused solely on demonstrating the ability to transfer genetic material to the myocardium. Locally enhanced expression of VEGF in the vicinity of ischemic myocardium improved collateral vessel density, regional blood flow, and myocardial function under stress conditions. The induction of new vessel growth by VEGF or its genetic progenitors is so promising that the Food and Drug Administration has recently approved a clinical trial proposed by Isner and his colleagues at the New England Medical Center. In their study, naked DNA coding for VEGF is to be infused into ischemic extremities unsuitable for surgical revascularization.

I have several questions for the authors:

Why did you select the 121 VEGF?

You noted minimal inflammation. Other investigators have consistently observed worrisome degrees of inflammation associated with Ad gene transfer. Why do you think inflammation was so minimal?

Why did you choose an Ad vector over naked DNA or plasmid-based gene transfer?

Have you performed experiments to determine the duration of gene expression or demonstrated VEGF within the myocardium?

Do you believe you have accelerated time-dependent collateral development or that you have induced a qualitative generation of neocollaterals?

Finally, do you think intravascular administration via the left anterior descending coronary artery would have had a comparable effect, inasmuch as this would allow a more convenient use of this technique if it were to become clinically applicable?

Dr. Mack. We chose the 121 isoform as opposed to the other isoforms because the 121 isoform is known to bind to just one of the VEGF receptors, unlike the other isoforms. Certain inflammatory cells such as monocytes and some carcinoma cell lines are known to express both or one of the VEGF receptors. We thought that the $VEGF_{121}$ isoform would provide us with the most specificity for endothelial cells in that it binds to the Flk-1 receptor and not to the Flt-1 receptor.

In addition, $VEGF_{121}$ does not have as much affinity for heparin as does $VEGF_{165}$ and would diffuse more into the ischemic milieu and therefore potentially enhance any therapeutic effect.

Concerning inflammation, contamination by replication-competent viral particles to a large extent determines the degree of inflammation when Ad vectors are used. The vector that we used was free of replication-competent particles up to 10^8 pfu. As Ad vector technology advances, the degree of inflammation potentially can become less and less.

Disadvantages of the Ad vector for many of the inherited diseases, in that it expresses the transgene for only a limited duration, is one of the main reasons why we chose an Ad for this application. It was our hypothesis that a localized, yet transient degree of expression would be just enough to induce a neovascular response. Any prolonged degree of expression raises the concern of disorganized angiogenesis, inappropriate angiogenesis at other sites, such as the retina, occult tumors, and arthritic joints. The fact that the first-generation Ad vectors are known to express the transgene only transiently is a true advantage for this application,.

Regarding the duration of expression, in this porcine model we have done only very preliminary studies looking at gene expression and quantifying gene expression. Prior work in our laboratory done by Christopher Magovern has demonstrated that an Ad vector expressing $VEGF_{165}$, expression can be as long as 7 to 14 days. Although we have not studied the duration of expression to date in the porcine model, we are planning to do that study and validate the fact that expression is transient.

Dr. Wechsler inquired about the mechanism contributing to our results, whether it is an increase in the endogenous response or a new generation of blood vessels. It is probably a combination of several things. The endogenous response probably is enhanced to some degree; however, other investigators, using VEGF and other growth factors and certain mitotic assays like bromodeoxyuridine and proliferating cell nuclear antigen, have demonstrated that a new generation of blood vessels develops. Therefore the collateral vessel response contributing to improved blood flow is likely a multifactorial process.

As far as the route of administration through the left anterior descending coronary artery, it was our purpose to directly inject the vector into the myocardium to avoid any systemic toxicity. Catheter-related devices that allow injection into the coronary vessel raise the concern of a systemic administration.

Dr. Margaret D. Allen *(Seattle, Wash.).* Did antibodies to Ad develop?

Dr. Mack. We looked at neutralizing antibodies at the time of Ameroid constrictor placement, the time of vector administration, and when the animals were put to death. Neutralizing antibodies to the Ad were observed at the time of sacrifice at a very low level. This has to be taken in context with what has been published for the most part in small animals, where the same dose of vector is given in an animal that is perhaps a thousand times smaller. The degree of neutralization in these experiments was relatively minimal.

Dr. Allen. That would be interesting, because it might affect retreatment in the future, for instance, for an ischemic patient who might need more than one treatment. It would be interesting to know whether the intramyocardial injection perhaps is less likely to induce an antibody response than an intravenous injection.

Dr. Mack. That is a very insightful point. Perhaps the route of administration does play a role here. As far as being able to administer the vector twice, there are 49 different Ad serotypes, and studies have been published whereby just altering the serotype can circumvent neutralizing immunity. This does not escape the cytotoxic T-cell response that clears the virus. However, clearly a repeat administration using an alternate serotype or immunomodulation can be achieved.

Mr. Reida M. El Oakley *(London, England).* I agree with Dr. Wechsler that your data would be more informative if you included two more control groups, one with the plasmid only and one with saline solution or nothing altogether.

Dr. Mack. We actually have in progress a treatment group in which the animals receive no injection at the second operation. Preliminarily, there appears to be no difference between that group and our AdNull-treated controls.